Reference: *Biol. Bull.* **195:** 282–289. (December, 1998)

Peptidergic Neurons in Barnacles: An Immunohistochemical Study Using Antisera Raised Against Crustacean Neuropeptides

S. G. WEBSTER

School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, UK

Abstract. Antisera raised against neuropeptides from decapod crustaceans were used to investigate whether balanomorph barnacles produce peptides analogous to those identified in some decapods. The distribution and structure of immunoreactive neurons was examined in *Balanus balanus, Balanus perforatus,* and *Chirona (Balanus) hameri* by whole-mount immunohistochemistry. In these species, no immunoreactivity was observed to antisera against CHH (crustacean hyperglycemic hormone), MIH (molt-inhibiting hormone), or RPCH (red-pigment-concentrating hormone), but neurons immunoreactive for pigment-dispersing hormone (PDH) and crustacean cardioactive peptide (CCAP) were observed. In all three species, PDH immunoreactivity was primarily associated with a pair of large (30–50 μm diam.) anterio-ventral perikarya in the ventral ganglion, projecting prominent axons along the great splanchnic nerves, which branched extensively in the segmental splanchnic nerves, directing several arborizing dendrites to the somatic extensor muscles. Occasionally, three pairs of anterio-dorsal perikarya were observed, which projected fine ipsilateral and contralateral axons along the great splanchnic nerves. A further 12 pairs of perikarya, apparently segmentally arranged, were observed in the thoracic ganglion. Several PDH-immunoreactive perikarya and associated branching plexus were observed in the supra-esophageal ganglion. CCAP immunoreactivity was mainly restricted to the ventral ganglion, where three pairs of perikarya (*ca.* 30–50 μm diam.) projected contralateral descending axons to the cirri. Occasionally a single pair of immunoreactive neurons were observed in the supra-esophageal ganglia. Although the anatomy of the CCAP-immunoreactive neurons in the ventral ganglion of barnacles might be homologous to conserved neural architectures in higher crustaceans, the anatomy of the PDH-immunoreactive neurons seems unique, and the morphology of the two large neurons in the ventral ganglion suggests a neuromodulatory role for this peptide, possibly associated with somatic extension.

Received 19 May 1998; accepted 10 September 1998.
E-mail: s.g.webster@bangor.ac.uk

Introduction

The morphology of the cirripede central nervous system is unique amongst arthropods in that it has undergone extensive modification associated with the sessile mode of life in the adult barnacle. Indeed, the *Bauplan* of the arthropod nervous system is scarcely discernible. The supra-esophageal ganglion is a simple bilobed structure with no external topographical features distinguishing a proto-, deutero-, or trito-cerebrum, and it appears to be principally involved in neural integration of information from the lateral and median photoreceptors. There is no trace of a sub-esophageal ganglion; the circumesophageal connectives join a ventral ganglionic mass that is sometimes referred to as the thoracic ganglion. There is no trace of an abdominal ganglion (Gwilliam and Cole, 1979).

Although much is known about the neurobiology of the barnacle CNS, particularly with regard to the photoreceptors and the neural networks involved in cirral movement (review by Gwilliam, 1987), very little is known about the nature or anatomy of peptidergic cells in the barnacle CNS. At the light microscopic level, Gomori-positive neurons have been observed in the supra-esophageal and ventral ganglion (Barnes and Gonor, 1958a,b; Van den Bosch de Aguilar, 1976, 1979). At the electron microscopic level, membrane-bound electron-dense vesicles reminiscent of neurosecretory granules have been

observed within axons in the median ocellar nerve of *Semibalanus cariosus* (Fahrenbach, 1965) and *Chirona (Balanus) hameri* (Clare and Walker, 1989); these vesicles apparently constitute a neurohemal area. Recently, Gallus *et al.* (1997) demonstrated the presence of FMRFamide immunopositive neurons in the ventral ganglion of *Balanus amphitrite*.

Because the morphology of the barnacle nervous system is so unusual, and because peptidergic neurons in those systems are so little known, the methods of immunohistochemistry were used to define and map neurons that might be producing peptides homologous to those now identified in some decapod crustaceans. From an evolutionary viewpoint, it was also of interest to determine whether peptidergic neuronal networks structurally homologous to those known in higher crustaceans could be identified in the greatly modified CNS of barnacles.

Materials and Methods

Animals and tissue preparation

Specimens of *Balanus balanus* were collected from rocks at extreme low-tide level at Church Island, Menai Strait, UK, and *Balanus perforatus* from rocks at mid-tide level at Concarneau, Brittany, France. *Chirona (Balanus) hameri* specimens were obtained by dredge from *Modiolus* beds off Langness, Isle of Man. Nervous systems were microscopically dissected under ice-cold saline, and since non-nervous tissue produces unacceptably high background staining, all of it was removed. Complete nervous systems were pinned with cactus spines to small pieces of Sylgard. Tissues were fixed for 8 h at 4°C in Stephanini's fixative (Stephanini *et al.*, 1967), or in 4% paraformaldehyde, 1% EDC (1-ethyl-3,3′-dimethylaminopropyl-carbodiimide) in 0.1 *M* phosphate buffered saline (PBS), pH 7.4, for 8 h at 0°C, washed in 0.1 *M* sodium phosphate buffer (pH 7.4) containing 0.5 *M* sucrose, and permeabilized by extensive (24 h) incubation in PBS containing 0.5% Triton X-100 containing 0.02% sodium azide (PTX).

Immunohistochemical techniques

Nervous systems were incubated in primary antibody, diluted in PTX, for 72 h. Antisera (and dilutions) used were (a) anti-molt-inhibiting hormone (MIH), *Carcinus* 1:500 (Dircksen *et al.*, 1988); (b) anti-crustacean hyperglycemic hormone (CHH), *Carcinus* 1:500 (Dircksen *et al.*, 1988); (c) anti-red-pigment-dispersing hormone (RPCH) 1:250 (Schooneveld and Veenstra, 1985); (d) anti-pigment-dispersing hormone (β-PDH) 1:1000 (Dircksen *et al.*, 1987); and (e) anti-crustacean cardioactive peptide (CCAP), 1:250 (Dircksen and Keller, 1988). After extensive washing in PTX (24 h), nervous systems were incubated in goat-anti-rabbit fluorescein isothiocyanate (GAR-FITC) (Sigma) 1:50 in PTX for 24–48 h, washed extensively in PTX, mounted in glycerol:PTX 1:1, and viewed under a fluorescence microscope. Permanent preparations were subsequently made by reincubation of tissues in goat-anti-rabbit IgG (Sigma) 1:100 (in PTX without sodium azide) (24 h), washing for 24 h in the same buffer, incubation in peroxidase-anti-peroxidase (PAP) (Sigma) 1:200 (24 h), and visualization with 3-3′-diaminobenzidine hydrochloride as detailed by Dircksen *et al.* (1991); this was followed by graded ethanol dehydration, clearing in methyl benzoate, and mounting in DPX.

Specificity tests (preabsorbtion controls) were performed for antisera that yielded positive results by incubating (24 h 4°C) 1 μl of antiserum with 10 nmol of the appropriate (synthetic) peptide dissolved in 10 μl PBS. After dilution, the antibody was then used for immunohistochemistry as detailed above.

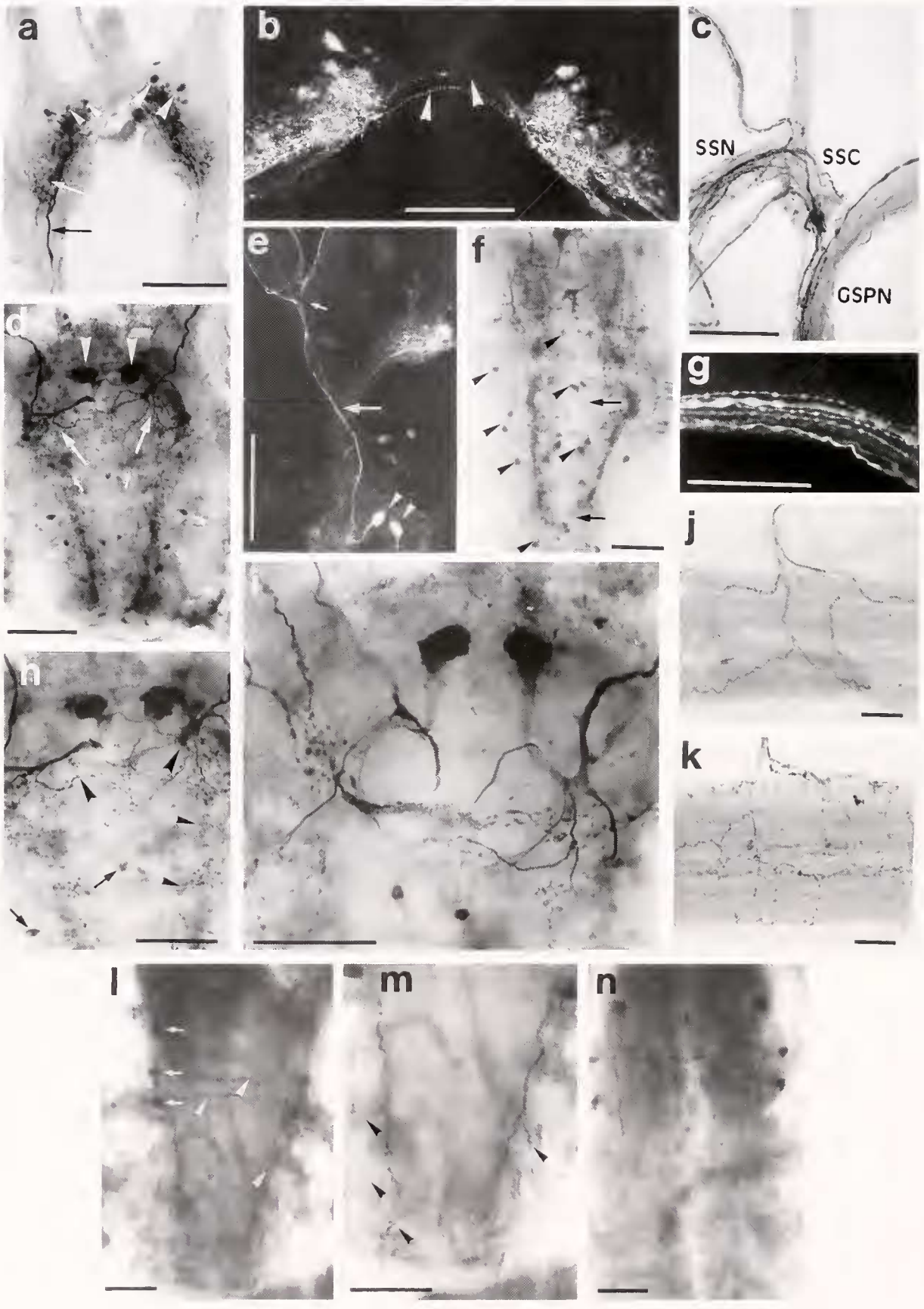
Results

Despite extensive investigation, immunopositive structures were never observed using MIH, CHH, or RPCH antisera. Nevertheless, PDH- and CCAP-immunoreactive (IR) neurons were consistently seen in preparations of *B. balanus*, *B. perforatus*, and *C. hameri*. Essentially, all species exhibited broadly similar PDH- and CCAP-IR structures; species differences were insignificant. Preabsorption controls completely abolished immunoreactivity.

PDH-immunoreactive neurons

The most striking and consistently observed immunoreactive structures seen in all species were a pair of large (30–50 μm diameter) anterio-ventral perikarya on the surface of the neuropil of the ventral ganglion (VG) (Fig. 1d–f, h, i; Fig. 2). These cells projected a large, conspicuous (often beaded) axon along the great splanchnic nerve (GSPN), branching extensively at the junction of this nerve with the segmental splanchnic nerve (SSN) (Fig. 1c). Additional branches from the axons ran to the posterior of the VG, and also to the midline, terminating in an extensive plexus (Fig. 1d, f, h; Fig. 2). Fine nerves from the SSN, each containing immunopositive dendrites, branched extensively over adjacent musculature; the attrahens and anterior prosomal muscles were particularly well innervated. When attrahens muscles were examined, a consistent pattern of immunopositive dendrites were seen (Fig. 1j, k).

In a few (*ca.* 5–10) preparations (particularly *C. hameri*), a group of three pairs of perikarya were seen on the anterio-dorsal surface of the VG. The anterior two pairs of perikarya projected fine axons ipsilaterally and contralaterally along the GSPN, whilst the posterior pair pro-


a
b
c
SSN
SSC
GSPN
d
e
f
g
h
j
k
l
m
n

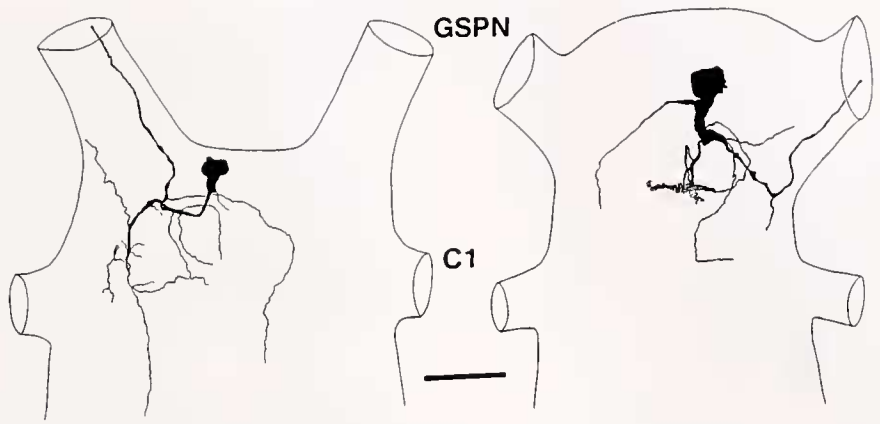


Figure 2. *Camera lucida* tracings of the large ventral PDH-immunopositive perikarya of the ventral ganglion. Left: *Balanus perforatus,* Right: *B. balanus.* GSPN = great splanchnic nerve, C1 = first cirral nerve. Scale bar = 100 μm.

jected axons ipsilaterally towards the GSPN, with a fine, posteriorly directed branch (Fig. 3). It should be emphasized that these neurons were not often visible in *B. perforatus* or *B. balanus,* but occasionally four immunopositive axons were observed in the GSPN. The large axon could invariably be traced to the anterio-dorsal perikarya mentioned earlier, but the fine axons (which exhibited prominent varicosities), could not be traced to the presumed group of three paired perikarya. Within the neuropil of the VG of *B. perforatus* and *B. balanus,* six pairs of small (15–20 μm diameter), weakly immunopositive, lateral perikarya projecting fine axons to the midline

Figure 1. Immunoreactive neurons in the barnacle central nervous system: (a–k) Pigment-dispersing-hormone immunoreactivity (PDH-IR); (l–n) crustacean-cardioactive-peptide immunoreactivity (CCAP-IR). All preparations are peroxidase-anti-peroxidase (PAP) except where noted. (a) The supra-esophageal ganglion of *Balanus perforatus,* showing two pairs of prominent perikarya (large arrowheads) and several less intensely stained, smaller perikarya (small arrowheads) in each hemisphere, arborizing dendrites and axons projecting along the circumesophageal connectives (arrows). (b) Fluorescein isothiocyanate (FITC) preparation of the supra-esophageal ganglion of *B. balanus,* showing the features seen in a, and also fine contralateral projections to each hemisphere (arrowheads). (c) PAP preparation of the origin of the segmental splanchnic nerve (SSN) of *B. balanus,* showing the extensive ramification of the single axon of the great splanchnic nerve (GSPN) in the segmental splanchnic connective (SSC). (d) Ventral ganglion of *B. balanus.* Note the two prominent perikarya (arrowheads), with prominent axons entering the great splanchnic nerve, and extensive dendrites (arrows). Small arrowheads indicate the small, weakly immunopositive neurons. Note the three pairs of perikarya (small arrows) out of the plane of focus. These are the dorsal perikarya, which are described in Fig. 3. (e) FITC preparation of the CNS of *B. balanus,* showing the two large perikarya in the ventral ganglion (arrowheads), directing axons along the great splanchnic nerve (small arrow), branching at the origin of the segmental splanchnic connective (small arrowhead). Note the intense immunoreactivity in the supra-esophageal ganglion (top right) in this preparation. (f) Ventral ganglion of *B. perforatus;* the large ventral perikarya are at the top of the figure. Note the small, weakly immunopositive lateral perikarya and median perikarya (arrowheads). The lateral perikarya project fine axons to the midline (arrows). (g) FITC preparation of the circumesophageal connective of *B. balanus.* The beaded axons are associated with the immunopositive neurons in the supra-esophageal ganglion, but could not be traced to the ventral ganglion. (h) Higher magnification of d, showing the large pair of ventral perikarya and branching dendrites (arrowheads). Note the small, weakly immunopositive median and lateral perikarya (arrows), and the extensive dendritic fields (small arrowheads) associated with branching descending fibers from the large ventral perikarya. (i) Fine structure of the pair of large ventral perikarya. (j–k) The attrahens muscles of *B. balanus* (upper) and *B. perforatus* (lower), showing branching immunopositive dendrites. (l) Ventral ganglion of *B. balanus,* showing three pairs of CCAP-immunoreactive perikarya (small arrows). The first pair are dorsal (out of the plane of focus); the posterior two pairs are ventral. Note the contralaterally projecting descending axons (arrowheads). (m) Higher magnification of l, showing last pair of perikarya at the top of the figure, detailing the descending axons, which branch into each cirral nerve (arrowheads). (n) Ventral ganglion of *C. hameri,* showing CCAP-immunoreactive cells and axons, with a morphology very similar to those seen in *B. balanus.* (The contralateral projections are broken along the midline in all preparations of this species, due to shrinkage.) Scale bars: a, b, c, f, h, l, m, n = 200 μm; d, g, j, k, i = 100 μm; e = 500 μm.

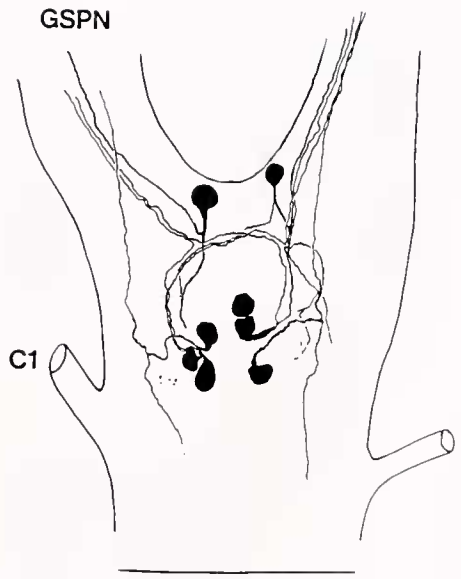


Figure 3. *Camera lucida* tracing of the ventral ganglion of *Chirona hameri*, detailing the gross structure of the large ventral PDH-immunopositive perikarya, and more detailed structures of the two groups (weakly immunoreactive) of three dorsal perikarya. The posterior cells direct axons ipsilaterally, whilst the anterior cells project axons ipsi- and contralaterally. Scale bar = 500 μ. Abbreviations as in Fig. 2.

were observed (Fig. 1f), and a further six pairs of very weakly immunoreactive perikarya were located centrally (Fig. 1d, f).

Apart from the above-mentioned structures, the only PDH-immunoreactive structures consistently seen were in the supra-esophageal ganglia of *B. balanus*, where at least two pairs of strongly immunoreactive and three pairs of weakly immunoreactive neurons were seen, associated with extensive dendritic fields with prominent varicosities on the surface and within the neuropil (Fig. 1a, b). Several contralaterally projecting fine axons were also observed (Fig. 1b). Occasionally, prominent beaded axons originating from the supra-esophageal ganglion were seen in the circumesophageal connectives (Fig. 1g), but these could never be traced to the ventral ganglion (Fig. 1e).

CCAP-immunoreactive neurons

CCAP-immunoreactivity was observed in all species of barnacles examined, although immunoreactivity was weak, and background staining was high, even when nervous systems were fixed in carbodiimide fixative (the fixative of choice for this peptide in whole mounts of arthropod nervous tissue (Dircksen *et al.*, 1991). For both *B. balanus* and *C. hameri*, similar morphologies of CCAP-immunoreactive neurons were observed (Fig. 1,1–n). The anterior pair of perikarya (*ca.* 50 μ diam.) are dorsal, the posterior two pairs (*ca.* 30 μ diam.) are ventral. All perikarya project a single axon contralaterally which descends to the posterior margin of the ventral ganglion with fine branches projecting along each cirral nerve. A detailed *camera lucida* reconstruction of these neurons (Fig. 4) shows the branching pattern of fine dendrites. In some preparations a pair of small (10–15 μm) neurons were seen in the supra-esophageal ganglia (the fine structure of these neurons was not investigated further).

For references, Figure 5 shows a schematic of the PDH- and CCAP-immunoreactive neurons observed.

Discussion

In the present study, several antisera raised to native peptides of decapod crustaceans were used to determine the neuroanatomy of peptidergic neurons in barnacles. Although a limited array of antisera were used, a notable finding was that CHH- and MIH-immunoreactive neurons were never observed in barnacle nervous systems. This might be explained by the considerable group- or even species-specificity of MIH and CHH (Keller, 1992). However, CHH-immunoreactive neurons have been identified in isopods (Nussbaum and Dircksen, 1995) and mapped in the cladoceran *Daphnia magna* and in an anostracan, *Artemia salina* (Zhang *et al.*, 1997). Thus, since CHH-like molecules seem to be a phylogenetically ancient group, the failure to observe CHH-like immunoreactivity in this study may well have been due to unsuitability of

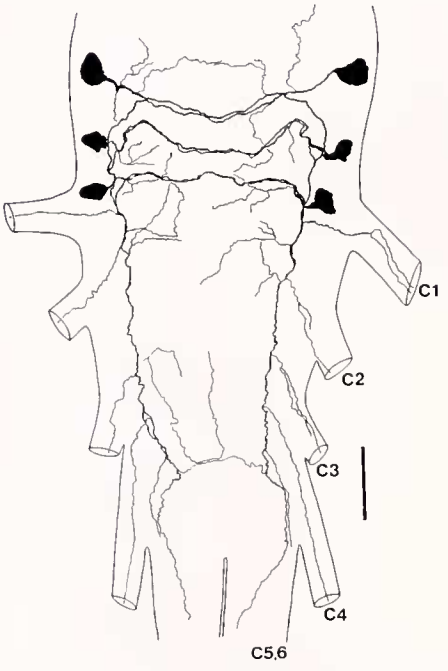


Figure 4. *Camera lucida* tracing of the CCAP-immunoreactive structures in the ventral ganglion of *Balanus balanus*. C1–6 = cirral nerves 1–6. Scale bar = 100 μ.

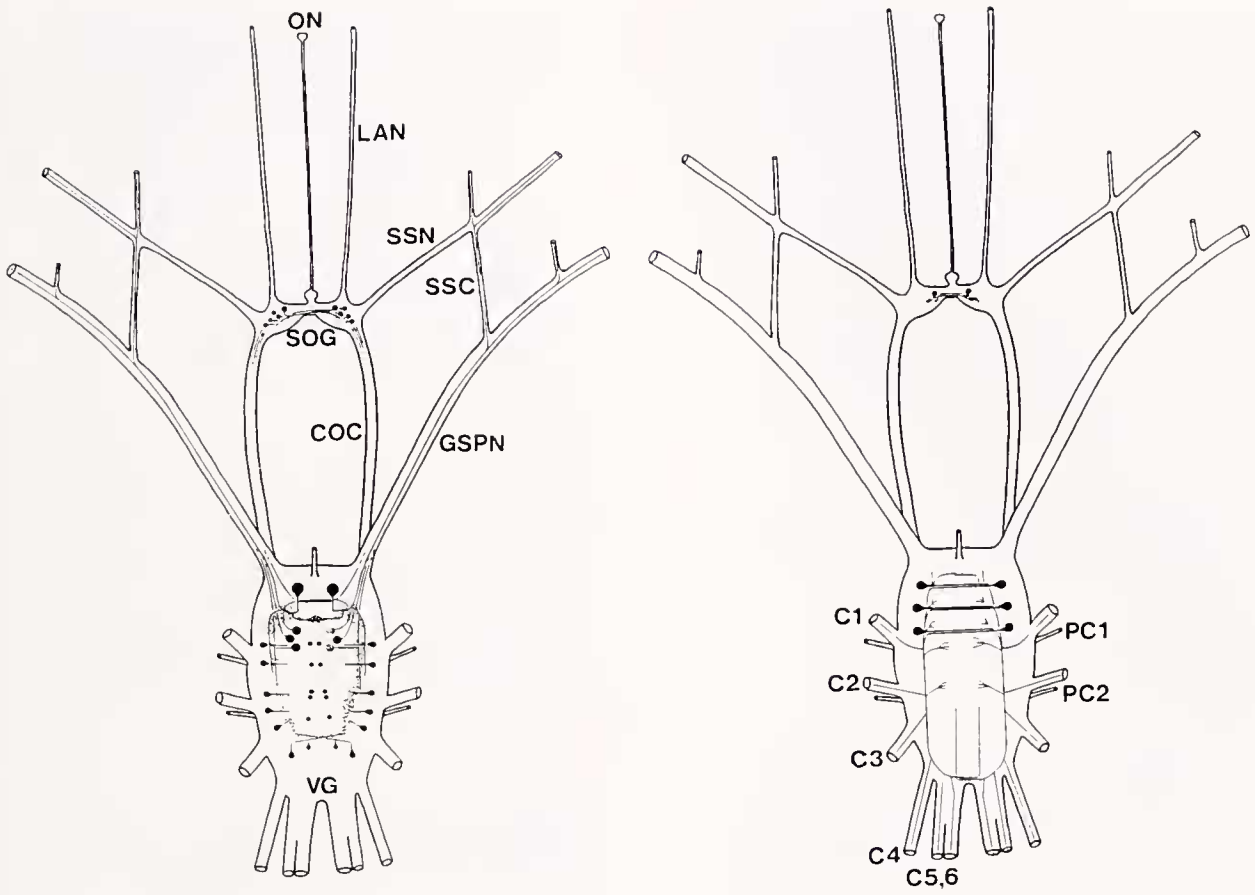


Figure 5. Schematic diagrams of PDH (left) and CCAP (right) immunoreactive neurons in the CNS of balanomorph barnacles. Abbreviations: C1–6: cirral nerves 1–6; COC: circumesophageal connectives; GSPN: great splanchnic nerve; LAN: lateral antennal nerve; OC: ocellar nerve; PC1–2: paracirral nerves 1–2; SOG: supra-esophageal ganglion; SSC: segmental splanchnic connective; SSN: segmental splanchnic nerve; VG: ventral ganglion.

the fixatives used, although the same fixative (Stephanini) was used in all studies mentioned here.

The absence of RPCH immunoreactivity is surprising. This peptide is a member of the ever-expanding adipokinetic hormone (AKH) group. Although many different AKHs have been identified in insects (See Gäde, 1997, for list), it appears likely that only one member, red-pigment-concentrating hormone (RPCH) occurs in crustaceans (Gaus *et al.*, 1990). It might be argued that the absence of RPCH (as a circulating neurohormone) would be expected since chromatophores are present only in malacostracans; however, immunocytochemical studies (Mangerich *et al.*, 1986; Nussbaum and Dircksen, 1995) using the same antiserum that recognizes the N-terminal tetrapeptide sequence common to many AKHs (Schooneveld and Veenstra, 1985) have shown that RPCH-immunoreactive interneurons are very widely distributed in the brain, thoracic and stomatogastric ganglia, and ventral nerve cord in malacostracans. Although RPCH from the central nervous system of decapods shows red-pigment-concentrating activity in bioassays (Fingerman and Couch, 1967), extracts of CNS from several species of barnacle result in pigment dispersion when injected into *Uca pugilator* (Sandeen and Costlow, 1961). This could indicate an absence of RPCH-like peptides in the CNS of barnacles, but it is more likely that the relative abundance of PDH-like material demonstrated in this study would override any pigment-concentrating effect of RPCH.

Although a pigment-dispersing hormone was first identified in crustaceans (Fernlund, 1976), immunocytochemical studies have not only verified a neurohormonal role for PDH, but have also documented a widespread occurrence in the CNS of crustaceans (Dircksen *et al.*, 1987; Mortin and Marder, 1991) and insects (Homberg *et al.*, 1991; Nässel *et al.*, 1991; Stengl and Homberg, 1994); it seems likely that PDH-like peptides, or more precisely, pigment-dispersing factors (PDFs) have a widespread,

perhaps universal, occurrence in arthropods (review by Rao and Riehm, 1993). Apart from the established role of PDH in controlling pigment migration in malacostracan crustaceans, the physiological significance of PDH-like peptides in arthropods has remained obscure. However, a role for PDH in control of circadian rhythmicity, proposed on the basis of the morphology of the immunoreactive neurons in the brain of orthopterans (Homberg *et al.*, 1991), has been confirmed (Stengl and Homberg, 1994); furthermore, some neurons that express the period (per) gene in the *Drosophila* brain also display PDH- immunoreactivity (Helfrich-Förster, 1995). In the present study, PDH immunoreactivity was seen in several types of neuron in the ventral and supra-esophageal ganglia of barnacles. The neuronal system, consisting of a single pair of large cells projecting axons along the GSPN, was extremely prominent. Apart from these PDH-immunopositive structures, the only invariably immunoreactive PDH cells appeared to be interneurons: the perikarya in the supra-esophageal ganglia projected axons along the circumesophageal connectives towards the ventral ganglion. Small perikarya in the VG appeared to be segmentally arranged—although the VG is fused, six pairs of perikarya correspond with the number of thoracic limbs; thus these cells may delineate neuromers. Additionally, the dendritic areas associated with the posteriorly directed branches of the two large PDH-IR perikarya appeared to show some arrangement reminiscent of a segmentally iterated pattern (Fig. 4d, h).

With respect to the possible functions of PDH-IR material in barnacles, the presence of PDH-IR in neurons terminating on the attrahens and anterior prosomal muscles is of interest. Cotransmission of peptides and neurotransmitters is a widespread, if not universal, phenomenon (review by Kupfermann, 1991). In crustaceans, a thoroughly investigated cotransmitter system is that of the crayfish tonic flexor muscle, which is innervated by five motoneurons, three containing proctolin, that potentiate tonic contraction of these muscles (Bishop *et al.*, 1987). It therefore seems possible that the two PDH-IR neurons innervating the somatic protractor may also have neuromodulatory roles in somatic extension in barnacles.

Crustacean cardioactive peptide (CCAP), originally isolated from pericardial organs of *Carcinus maenas* (Stangier *et al.*, 1987), is another example of a neuropeptide with a wide, if not universal, occurrence in arthropods. The conserved neuronal networks of this peptide in arthropods have recently been reviewed by Dircksen (1998). Although the best known action of this peptide is its cardioacceleratory action, related (myotropic) actions in crustaceans include acceleration of scaphognathite rate and increased hindgut motility (Stangier, 1991). An important recent finding is that CCAP is a potent modulator of the pyloric rhythm of the stomatogastric ganglion of the crab *Cancer borealis* (Weimann *et al.*, 1997). Immunocytochemical studies on the isopod *Oniscus asellus* (Nussbaum *et al.*, 1995) suggest that CCAP might be involved in the biphasic exuviation pattern seen in isopods. For insects, broadly comparable myotropic roles have been observed, and it is expected (from morphology of CCAP-IR neurons that a multiplicity of functions have yet to be determined (Dircksen, 1998). In the present study, three pairs of CCAP-IR neurons observed in the ventral ganglion were notable. The perikarya essentially projected descending contralateral axons towards the cirri, but were too fine to allow the complete neural pathway to be traced. Although backfills have not demonstrated that barnacles have a neuronal architecture equivalent to that of higher crustaceans (Gwilliam and Cole, 1979; Gwilliam, 1987), the arrangement of the CCAP-IR neurons in the barnacle ventral ganglion is broadly reminiscent of the arrangement of the cdn-type-2 neurons in the thoracic ganglion of decapod crustaceans (Dircksen, 1998) in the sense that they project contralateral descending axons. Further speculation with regard to homology or function would, however, be premature.

Acknowledgements

This work was supported by a Royal Society research grant. I am indebted to Dr. Heinrich Dircksen, University of Bonn, for providing antisera, and especially for his helpful advice and discussion. This paper is dedicated to the memory of the late Professor Dennis Crisp, F.R.S., who initiated and stimulated my interest in barnacle biology with his enthusiasm.

Literature Cited

Barnes, H., and J. J. Gonor. 1958a. Neurosecretory cells in some cirripedes. *Nature* **181:** 194.

Barnes, H., and J. J. Gonor. 1958b. Neurosecretory cells in the cirripede, *Pollicipes polymerus* J. B. Sowerby. *J. Mar. Res.* **17:** 81–102.

Bishop, C. A., J. J. Wine, F. Nagy, and M. R. O'Shea. 1987. Physiological consequences of peptide cotransmission in a crayfish nerve muscle preparation. *J. Neurosci.* **7:** 1767–1779.

Clare, A. S., and G. Walker. 1989. Morphology of the nervous system of barnacles: the median ocellus of *Balanus hameri* (= *Chirona hameri*). *J. Mar. Biol. Assoc. UK* **69:** 769–784.

Dircksen, H. 1998. Conserved crustacean cardioactive peptide (CCAP) neuronal networks and functions in arthropod evolution. Pp. 302–333 in *Recent Advances in Arthropod Endocrinology*, G. M. Coast and S. G. Webster, eds. Cambridge University Press.

Dircksen, H., and R. Keller. 1998. Immunocytochemical localization of CCAP, a novel crustacean cardioactive peptide, in the nervous system of the shore crab, *Carcinus maenas* L. *Cell Tiss. Res.* **254:** 347–360.

Dircksen, H., C. A. Zahnow, G. Gaus, R. Keller, K. R. Rao, and J. P. Riehm. 1987. The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands: identification by an antiserum against synthetic PDH. *Cell Tiss. Res.* **250:** 377–387.

Dircksen, H., S. G. Webster, and R. Keller. 1988. Immunocytochemi-

cal demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. *Cell Tiss. Res.* **251:** 3–12.

Dircksen, H., A. Müller, and R. Keller. 1991. Crustacean cardioactive peptide in the nervous system of the locust, *Locusta migratoria:* an immunocytochemical study on the ventral nerve cord and peripheral innervation. *Cell Tiss. Res.* **263:** 439–457.

Fahrenbach, W. H. 1965. The micromorphology of some simple photoreceptors. *Z. Zellforsch.* **66:** 233–254.

Fernlund, P. 1976. Structure of a light-adapting hormone from the shrimp *Pandalus borealis. Biochim. Biophys. Acta* **439:** 17–25.

Fingerman, M., and E. F. Couch. 1967. The red pigment dispersing hormone of the abdominal nerve cord and its contribution to the physiology of the prawn *Palaemonetes vulgaris. Rev. Can. Biol.* **26:** 109–117.

Gäde, G. 1997. The explosion of structural information on insect neuropeptides. *Prog. Chem. Org. Nat. Prod.* **71:** 1–128.

Gallus, L., A. Diaspro, R. Rolandi, M. Fato, and G. Taglialierro. 1997. Three-dimensional reconstruction of FMRF-amide immunopositive neurons in the ventral ganglion of the barnacle *Balanus amphitrite* (Cirripedia, Crustacea). *Eur. J. Histochem.* **2:** 99–100.

Gaus, G., L. H. Kleinholz, G. Kegel, and R. Keller. 1990. Isolation and characterization of red-pigment-concentrating hormone (RPCH) from six crustacean species. *J. Comp. Physiol. B* **160:** 373–379.

Gwilliam, G. F. 1987. Neurobiology of barnacles. Pp. 191–211 in *Barnacle Biology,* A. J. Southward, ed. Balkema, Rotterdam.

Gwilliam, G. F., and E. S. Cole. 1979. The morphology of the central nervous system of the barnacle, *Semibalanus cariosus* (Pallas). *J. Morphol.* **159:** 297–310.

Helfrich-Förster, C. 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA* **92:** 612–616.

Homberg, U., S. Wurden, H. Dircksen, and K. R. Rao. 1991. Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. *Cell Tiss. Res.* **266:** 343–357.

Keller, R. 1992. Crustacean neuropeptides: structures, functions and comparative aspects. *Experientia* **48:** 439–448.

Kupfermann, I. 1991. Functional studies of cotransmission. *Physiol. Rev.* **71:** 683–732.

Mangerich, S., R. Keller, and H. Dircksen. 1986. Immunocytochemical identification of structures containing putative red pigment-concentrating hormone in two species of decapod crustaceans. *Cell Tiss. Res.* **245:** 377–386.

Mortin, L. I., and E. Marder. 1991. Differential distribution of (β-PDH)-like immunoreactivity in the stomatogastric nervous system of five species of decapod crustaceans. *Cell Tiss. Res.* **265:** 19–33.

Nässel, D. R., S. Shiga, E. M. Wikstrand, and K. R. Rao. 1991. Pigment-dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual system. *Cell Tiss. Res.* **266:** 511–523.

Nussbaum, T., and H. Dircksen. 1995. Neuronal pathways of classical crustacean neurohormones in the central nervous system of the woodlouse, *Oniscus asellus* (L.). *Phil. Trans. R. Soc. Lond. B* **347:** 139–154.

Rao, K. R., and J. P. Riehm. 1993. Pigment-dispersing hormones. *Ann. NY Acad. Sci.* **680:** 78–88.

Sandeen, M. I., and J. D. Costlow. 1961. The presence of decapod pigment-activating substances in the central nervous system of representative Cirripedia. *Biol. Bull.* **120:** 192–205.

Schooneveld, H., and J. A. Veenstra. 1985. Insect neuroendocrine cells and neurons containing various adipokinetic hormone (AKH)-immunoreactive substances. Pp. 425–434 in *Neurosecretion and the Biology of Neuropeptides,* M. Kobayashi, H. A. Bern, and A. Urano, eds. Japan. Sci. Soc. Press, Tokyo; Springer, New York.

Stangier, J. 1991. Biological effects of crustacean cardioactive peptide, a putative neurohormone/neurotransmitter from crustacean pericardial organs. Pp. 201–210 in *Comparative Aspects of Neuropeptide Function,* G. B. Stephano and E. Florey, eds. Manchester University Press, UK.

Stangier, J., C. Hilbich, K. Beyreuther, and R. Keller. 1987. Unusual cardioactive peptide (CCAP) from pericardial organs of the shore crab *Carcinus maenas. Proc. Natl. Acad. Sci. USA* **84:** 575–579.

Stengl, M., and U. Homberg. 1994. Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J. Comp. Physiol. A* **175:** 201–213.

Stephanini, M., C. De Martino, and L. Zamboni. 1967. Fixation of ejaculated spermatozoa for electron microscopy. *Nature* **216:** 173–174.

Van den Bosch de Aguilar, Ph. 1976. Etude histochemique du système neurosécréteur de *Balanus perforatus* et *B. balanoides* (Crustacea; Cirripedia). *Gen. Comp. Endocrinol.* **30:** 228–230.

Van den Bosch de Aguilar, Ph. 1979. Neurosecretion in the Entomostraca crustaceans. *Cellule* **73:** 29–48.

Weimann, J. M., P. Skiebe, H. G. Heinzel, C. Soto, N. Kopell, J. C. Jorge-Riviera, and E. Marder. 1997. Modulation of oscillator interactions in the crab stomatogastric ganglion by crustacean cardioactive peptide. *J. Neurosci.* **17:** 1748–1760.

Zhang, Q., R. Keller, and H. Dircksen. 1997. Crustacean hyperglycaemic hormone in the nervous system of the primitive crustacean species *Daphnia magna* and *Artemia salina* (Crustacea: Branchiopoda). *Cell Tiss. Res.* **287:** 565–576.